Task-Specific Regulation of Microglial Reactivity and Blood Brain Barrier Permeability

Undergraduate Research Thesis

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Abstract

Spinal cord injury (SCI) can have devastating results, including debilitating loss of mobility. In human SCI patients, locomotor training on a level treadmill is often used to improve motor function. Animal studies investigating more effective therapies have shown downhill training to enhance recovery [1]. This effect is due to increased delivery of eccentric sensory feedback to the central nervous system (CNS). An eccentric contraction is a controlled lengthening contraction where muscles met with resistance send signals to the CNS that determine the amount of muscle output. Downhill treadmill exercise increases levels of eccentric feedback and has been shown to improve walking ability after SCI, producing gait patterns that nearly match naïve animals [1]. Eccentric feedback can be delivered in varying dosages and intervals, similar to a drug. Dose can be modulated utilizing different treadmill elevations that provide minimal (uphill) and maximal (downhill) amounts of eccentric feedback at walking and running speeds. It remains unclear how task-specific eccentric exercise differentially impacts the microenvironment within the spinal cord. Here, we test the hypothesis that different forms of sensory feedback produced during task-specific training paradigms differentially influence blood spinal cord barrier (BSCB) permeability and microglial reactivity. In order to identify what changes result from task-specific exercise training, mice with intact nervous systems were utilized in this study. Nineteen uninjured C57BL/6 mice were randomized into 5 groups: downhill walk (n=4), downhill run (n=3), uphill walk (n=4), uphill run (n=4), and unexercised sedentary (n=4). Microglial reactivity was measured in the spinal cord with iba-1 labeling. A vascular tracer, Evans blue dye (EBD), was used to measure BSCB stability. Analysis of these two outcome measures unexpectedly revealed uninjured exercised mice to display evidence of BSCB permeability in lamina X and the intermediate lamina (lamina VII), with greatest permeability in downhill walking animals. We hypothesize that task-specific exercises causes BSCB permeability by increasing angiogenesis within lamina X, a region known to be involved in incline walking as well transmitting nociceptive and mechanoreceptive information, and the intermediate lamina, the location of locomotor central pattern generators (CPG) [2-5]. Evidence of vessel growth was analyzed using Ly6C immunohistochemical staining of vasculature in lumbar tissue. Results of this study can be used to optimize exercise therapies for SCI patients.

Introduction

Spinal cord injury (SCI) has devastating effects on the body that can dramatically impact an individual's everyday living. Muscle spasticity, autonomic dysreflexia, and loss of bladder control are but a few symptoms seen after a SCI. Loss of motor function is perhaps the most debilitating result of SCI and is the target of many research studies. These motor deficits are the product of several injury mechanisms that are initiated by damage to the spinal cord. Primary injury mechanisms are those that occur at the time of the injury [6]. The force applied to the spinal cord results in an area of damaged tissue composed of severed axons and broken blood vessels. After the initial impact of the SCI, secondary injury mechanisms begin as a result of the body's response to the damaged tissue [6]. These are comprised of pathways such as blood spinal cord barrier (BSCB) permeability, microglial reactivity, cell death, ischemia, and cytokine release. Secondary injury mechanisms are a focus of research due to their potential to affect recovery. By understanding the mechanisms of these secondary injuries, more refined treatment options can be created to prevent further pathology.

The microenvironment of the spinal cord after an SCI changes as a result of secondary injury mechanisms. Shortly after a SCI, these environmental changes allow an increase in axonal sprouting around the lesion site [7, 8]. This window of spinal plasticity can be harnessed to further improve motor function. It has been found that delivering exercise training can encourage reorganization as well as axonal sprouting within the spinal cord [9]. By utilizing exercises that are task-specific, such as treadmill training, a focus can be placed on activating and strengthening particular pathways of interest. In order to maximize motor recovery, afferent signaling must be used to activate neuronal circuits in a manner similar to everyday walking [10]. Specificity of treadmill exercise can be altered by changing the degree of incline as well as modulating the intensity of exercise. By utilizing downhill treadmill training, levels of afferent signaling are emphasized through eccentric muscle contraction. In eccentric muscle contraction, afferent input is used to determine the level of motor output [11]. Due to the increased eccentric demand seen in downhill exercise, greater levels of afferent input are directed to those spinal circuits. The amount of afferent input can further be modified by the speed of the treadmill. By increasing the speed and the number of steps taken per minute, the intensity of afferent drive to the neural networks is also increased.

The secondary injury mechanisms involved after a SCI create further damage to the spinal cord. Microglia are resident immune cells within the central nervous system (CNS), and play a large role in the inflammatory response. In an intact nervous system, microglia have a phenotype consisting of a small round soma, surrounded by thin processes radiating from it. They play a role in functions such as axonal growth, phagocytosis, and cell signaling [12]. When SCI occurs, these cells become activated, pulling in their processes and expanding in size. They begin to secrete neurotoxic molecules and cytokines that are damaging to the spinal cord [13]. It has been found that these harmful activated cells can be found 10 segments or more below the lesion [14, 15]. When treadmill training is applied after a SCI, it remains unknown if the changes seen in microglia are due to the SCI damage, or the exercise therapy delivered.

The role of exercise in BSCB permeability also remains unclear. The CNS is protected by layers of specialized cells that prevent the passage of large molecules such as neurotoxic chemicals and viruses. In the CNS blood vessels are formed by endothelial cells held together by tight junctions. Pericytes lie on top of the endothelial cells and help to determine BSCB formation. Astrocytic end feet attach to this layer and have been found to regulate the barrier's strength [16]. Together these endothelial cells and astrocytes form the BSCB. In SCI, this barrier is disrupted as a result of damage to the blood vessels, allowing molecules from outside the CNS to enter. Strength and regulation of the BSCB in response to exercise has not yet been investigated, raising the question if exercise in itself could alter BSCB permeability.

During development, the CNS undergoes rapid expansion and maturation. Alongside neuronal growth and myelination, heightened levels of angiogenesis play an important role in development of the CNS. Angiogenesis, the growth of new blood vessels, can also be promoted in neurodegenerative diseases and tumor growth [16]. It can be regulated by several factors that can either increase or decrease the formation of new vessels. Factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor, and interleukins can promote vascular growth, while avastin, angiostatin, and endostatin inhibit angiogenesis [17, 18]. New vasculature takes time to form and fully mature. During the process of angiogenesis, vasodilation occurs, thereby weakening the barrier around the vasculature and making it more permeable to molecules entering from the periphery [19]. Preliminary data shows increased permeability of the BSCB around lamina X of the spinal cord.

In a transverse section of the spinal cord, the grey matter can be broken down into 10 distinct regions defined by their cellular structure [20]. These regions, referred to as lamina,

comprise several well-known regions of the spinal cord; such as the ventral, intermediate and dorsal horns. Lamina X is composed of several cell layers surrounding the central canal. It consists of 3 distinct zones. Most proximal to the central canal is an ependymal cell layer that continues into lamina X. Moving distally is the subependyma zone, composed of various glial cells making up the largest layer of lamia X. The final, most distal zone from the central canal is the neutrophil zone. This zone contains elements such as dendrites, axons, terminals, astrocytes and neurons [21]. Lamina X contains axons that play an important role in transmitting afferent mechanoreceptive and nociceptive information [3-5, 22]. This region has also shown an increase in neural activity during walking exercise [2]. Nociceptive pathways through lamina X may receive increased levels of afferent signaling and require additional vascular support during different forms of exercise. The intermediate lamina, lamina VII, is best known for its location of central pattern generators (CPG). The CPGs are a collection of neurons and interneurons that can produce rhythmic movements, such as walking, without descending input from the brain. This unique mechanism makes it a target for SCI recovery research. If these CPGs are highly active during task-specific exercise, the intermediate lamina may also require additional vascularization to support the increased activity of these cells.

There have been a multitude of studies conducted using exercise to take advantage of neuronal plasticity after SCI [23-25]. How to make these therapies more successful is still under investigation. Despite the wide use of exercise as a therapy for SCI, the knowledge of how these therapies impact an intact nervous system is not fully understood. This makes it difficult to discern if the changes we are seeing in CNS are a result of the SCI or the exercise itself. In this study, we examine how exercise alone, in an uninjured spinal cord, may differentially impact microglia reactivity, BSCB permeability, and angiogenesis. We hypothesize that the specificity of exercise training may distinctively alter the permeability of the BSCB and reactivity of microglia . Changes seen in the permeability of the BSCB may be attriubutible to angiogenesis within the region.

Materials and Methods

Subjects and groups

This study utilized uninjured, female C57BL/6 mice (n=19). The mice were randomly divided into 5 groups using an online random sequence generator. One group remained sedentary (n=4) while the remaining 4 groups were assigned to specific exercise procedures: downhill running (DHR, n=3), downhill walking (DHW, n=4), uphill running (UHR, n=4), and uphill walking (UHW, n=4). Animals were housed 4 mice per cage with ad libitum access to food and water and exposed to a 12-hour light/dark cycle. Animal care, housing, and training was completed in accordance with The Ohio State University Laboratory Animal Care and Use Committee.

Pre-exercise training

All exercise groups were acclimated to the treadmill prior to the start of their specific training. This treadmill exposure took place over the course of 3 days, for a total of 25 minutes. The introduction of mice to the treadmill began on a non-moving, flat device, followed by a moving treadmill belt with speeds ranging from 5.5 to 10 m/min. An assessment of the animal's walking was done using the Basso Mouse Scale for Locomotion (BMS). All animals were found to have no pre-

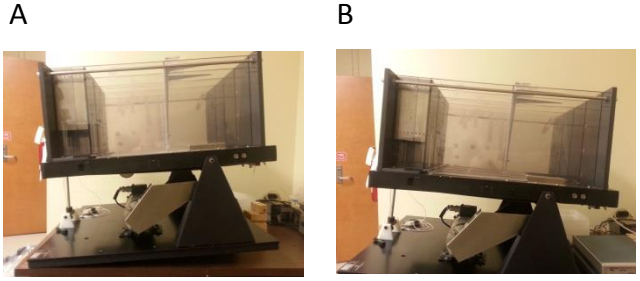

Figure 1. *Downhill exercised groups were trained on a decline of 5.7^o (**A**) while uphill exercise was delivered on a treadmill inline of 14^o (**B**).*

existing conditions and normal gait deeming them eligible to enter the study.

Training paradigm

Animals within the exercise groups were treadmill trained for 20 minutes a day for 6 continuous days. This was done in two 10 minute intervals with a 10 minute resting break in between each treadmill bout. Uphill exercise groups were placed on a treadmill at a 14^{o} incline, while animals in the downhill exercise groups were trained on a 5.7^{o} declined treadmill (figure 1). In running groups, treadmill speed began at 10 m/min the first day and was successively increased by a maximum of 2 m/min on a daily basis. All running animals reached a final treadmill speed of 16 m/min on the final day of exercise. Animals in the walking groups maintained a 7 m/min speed throughout the duration of the exercise training.

Dye Administration and Tissue Collection

Prior to perfusion, mice received an intraperitoneal injection of Evans blue dye (EBD) in normal saline (1.2%, 4mL/kg) in order to assess BSCB permeability [26]. After injection, the dye was allowed to circulate for 30 minutes at which time anesthetic was administered (ketamine 138 ml/kg, xylazine 20 ml/kg) (figure 2). Animals were transcardially perfused using phosphate buffered saline (0.1M) followed by paraformaldehyde (4%). The brain and entire spinal cord was then collected. Samples were then cryoprotected in sucrose (30%), frozen in M-1 mounting media (Thermo Scientific, Waltham, MA), and cut in 20 micrometer transverse sections.

Figure 2. *EBD administration results in blue skin coloration in mice due to its adhesion to blood albumin.*

Histology

Ionized calcium-binding adaptor molecule 1 (Iba-1) and lymphocyte antigen 6 complex C (Ly6C) labeling were implemented using protocols from prior studies. The labeling of microglia with iba-1 took place over the course of two days. Tissue was warmed to room temperature for two hours then rinsed 3x 5 minutes. Sections were then blocked for 2 hours in a solution consisting of 1%BSA, 0.1%FG, 3%NGS, 0.2%Tx-100, and PBS. Rabbit anti-Iba primary in a 1:200 concentration with block was applied to the tissue and stored overnight at 4° C. PBS was used to rinse the tissue 3x 5 minutes prior to the application of goat anti-rabbit fluorescent secondary. Secondary remained on the spinal sections for 1 hour. They were then rinsed 2x 5 minutes with PBS prior to cover slipping.

Ly6C was used to label vasculature within the spinal sections. The tissue was thawed to room temperature for 1 hour and rinsed 2x 5 minutes in PBS + 1% BSA. Blocking solution was created with 1% BSA and 2% NGS in PBS and left on the tissue for 1 hour. Sections were rinsed 2x5 min in PBS + 1%BSA before rat anti-mouse primary was applied to the tissue (Abcam 15627 Rat mAB Ly6C). Primary incubated for 48 hours at 4° C. Sections were washed again 2x 5 min in PBS + 1%BSA prior to fluorescent secondary application (A594 anti-rat). Secondary remained on the tissue for 24 hours at 4° C. A final rinsing step 2x 5 minutes in PBS + 1%BSA was done prior to cover slipping.

Confocal Microscopy

All images of Iba-1 labeling, Ly6C labeling, and EBD were collected using fluorescent confocal microscopy (Olympus Filter FV1000 confocal Microscope).

Proportional Area analysis

Proportional area of Iba-1 labeled microglial reactivity, Ly6C labeled vasculature, and EBD permeability was measured utilizing ImageJ software. Proportional area analysis was executed using images of lumbar cord taken at 10X. Using

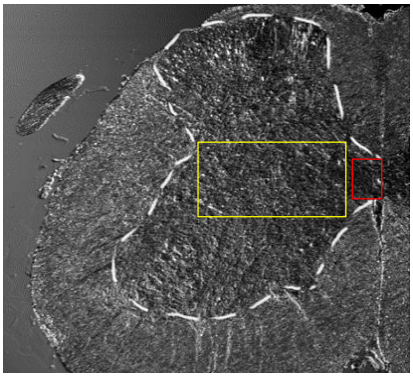

Figure 3*. ImageJ software was used to assess the proportional area of positive labeling by enclosing the intermediate lamina (yellow) and half of lamina X (red) in two separate boxes.*

ImageJ, a box was used to outline lamina VII and X, then the percent area of positive labeling within the box was measured (figure 3). The size of the box remained consistent throughout analysis for each region, with their size determined by the average size of lamina VII and X images. Boxes were placed in a best fit manner within the region of interest. In the analysis of lamina X, a box was placed to cover a hemisection of the lamina. The medial aspect of the box was aligned with the midline of the spinal cord section. Box placement around lamina VII was consistently placed to be centered within the region. Proportional area measurements of lamina X and VII were completed on the same side of the spinal cord in Ly6C and EBD analysis. Images were set at an ideal threshold level per image to ensure analysis of accurate labeling. Microglia reactivity was determined by utilizing high resolution images of a single microglia within lamina VII and measuring its length with imageJ (figure 4). All imageJ assessment methods remained consistent between each analysis.

Statistical Analysis

A one way ANOVA was used to assess the statistical significance between animal groups. Significance was determined at $p<0.05$. The standard error of the mean (SEM) is reported as a measure of variance.

Results

Increase in Microglial Reactivity

Proportional area analysis was used as a primary assessment of the microglial response to exercise (Figure 4). Within lamina X, the extent of Iba-1 labeling remained relatively consistent across all exercised and unexercised groups, with mean levels ranging from 2.31% to 4.4% (mean area: DHR 2.86±0.76%, DHW 4.39±0.37%, UHR 3.96±1.73%, UHW 4.40±1.40%, Sedentary 2.31±0.38%). This uniformity did not continue into the intermediate lamina. The proportional area of microglia within the intermediate lamina of DHW animals was significantly higher than that of sedentary controls (mean area: 4.32±0.88% in DHW and 1.33±0.22% in Sedentary animals; $p<0.05$). The lowest proportional area was seen in the sedentary group. The DHR, UHR, and UHW groups maintained similar levels of Iba-1 labeling with mean areas of 2.86±0.29%, 3.08±0.74%, and 3.04±0.46% respectively.

Normal Microglial Phenotype seen in the Intermediate Lamina

Length of a microglial cell body within the intermediate lamina was used to determine the cell's phenotypic changes. Phenotypic analysis was done on 100x images (figure 4). Cell bodies between groups were found to be of similar lengths, with group mean lengths ranging from 8.47 μm in the UHR group to 11.48 μm in the sedentary group (mean length: DHR 8.75±1.25 μm, DHW 11.10±2.68 μm, UHR 8.47±0.90 μm, UHW 9.26±1.25 μm, Sedentary 11.48±0.90 μm)

Exercise Increases BSCB Permeability

The degree of BSCB permeability within the lumbar cord was assessed using proportional area analysis. A baseline level of normal lamina X permeability was established at an average of 0.07% in sedentary control animals (± 0.04%). Analysis of lamina X revealed exercised animals to have substantially increased levels of BSCB permeability when compared to sedentary

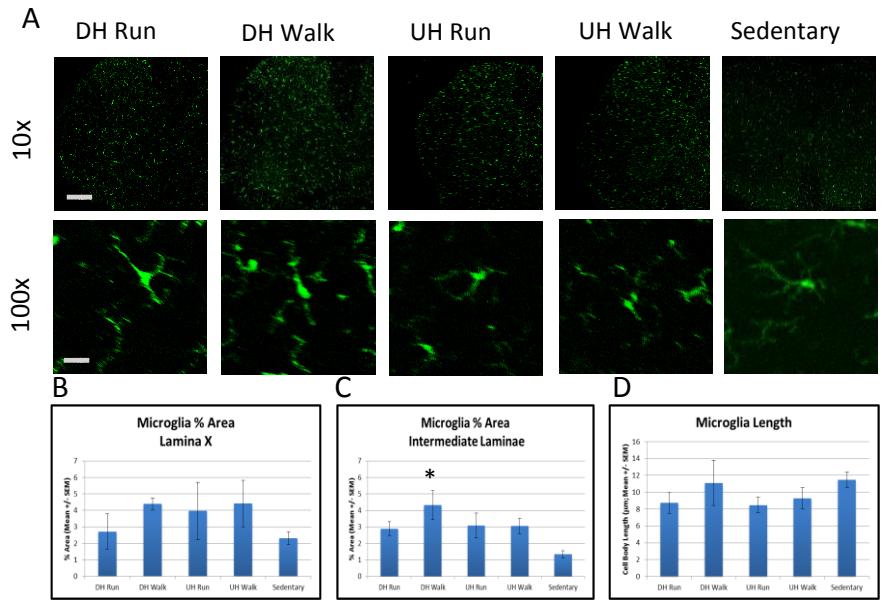

Figure 4. *Iba-1 labeling revealed an exercise induced increase in the proportional area of microglia in lamina VII and X (**B**, **C**). A significant increase was found in the DHW group when compared to the sedentary control animals (p< 0.05) (**C**). Little change was seen in the cell body length of microglia between groups (**D**). Scale bars: 10x; 200 mm, 100x; 20 μm.*

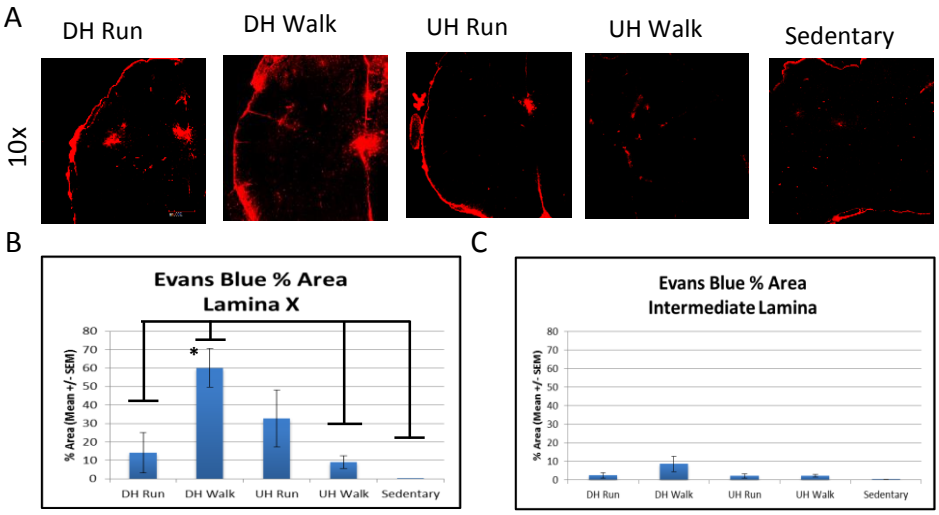

Figure 5. *A proportional area increase was found in both the intermediate lamina and lamina X in response to exercise. Low levels were seen in exercised groups in the intermediate lamina (**C**). A larger increase in BSCB permeability was seen exercised groups in lamina X. The DHW group showed a significant increase when compared to DHR, UHW, and sedentary groups (**B**) (p < 0.05). Scale bars: A; 200 μm*

controls (figure 5). The DHW group showed a significantly heightened permeability of 60.07% when compared to DHR, UHW, and Sedentary animals based on a one-way ANOVA analysis (mean area: DHR 35.60±22.84%, DHW 60.07%±10.47%, UHW 9.15±3.45%, Sedentary 0.07±0.42%; $p < 0.05$). The UHR group also showed a substantial amount of permeability with 32.61% of lamina X permeated with EBD (mean area: UHR 32.60±15.38%). The DHR and UHW groups presented similar low levels of permeability at 14.13% and 9.11% respectively. Within the intermediate lamina all exercised animals were found to have a minor increase EBD extravasation

that was not significant. DHW animals showed the most permeability at 8.52%; while DHR, UHR, and UHW animals indicated lower levels around 2%. These changes were not found to be significantly different from sedentary controls (mean area: DHR 14.12±11.80%, DHW 8.52±4.07%, UHR 2.09±1.15%, UHW 2.06±0.75%, Sedentary 0.27±0.12%).

Concentration of Vasculature Suggests Angiogenesis

Proportional area analysis of the Ly6C labeling within lamina X revealed a density increase in vasculature in response to exercise (figure 6). The DHW group exhibited the largest percentage of vasculature within lamina X at 13.13%, a significant increase when compared to sedentary controls (mean area: 13.13±2.13% in DHW group and 6.38±0.92% in Sedentary animals; $p<0.05$). The remaining DHR, UHR, and UHW groups maintained similar vascular levels ranging from 6.95% to 8.56% (mean area: DHR 6.95±0.83%, UHR 7.96±1.74%, UHW 8.56±2.29%). Within the intermediate lamina UHR, UHW, and Sedentary animals contained comparable percentages of blood vessels, around 7% (mean area: UHR 7.30±0.66%, UHW 7.89±13.6%, Sedentary 7.94±1.66%). DHR and DHW had more substantial increases in vasculature at 9.99±0.77% and 11.72±2.70%. These differences were not found to be significant.

Discussion

This study shows for the first time that exercise itself impacts the microenvironment of the CNS. The proportional are of microglia within lamina X showed little difference between groups, while a significant change was seen in the intermediate lamina. The DHW group displayed significantly higher levels of microglia than sedentary animals within the intermediate lamina. This difference in microglia indicates that exercise is effecting the microglia more significantly in lamina VII than in lamina X, and that the specificity of the exercise plays a role in what regions of the spinal cord microglial changes are observed.

After a SCI, a phenotypic change in the microglia occurs and they become activated. These activated microglia release harmful cytotoxic inflammatory molecules and have cell bodies that are much larger than in un-activated cells. The processes projecting off the cell body are pulled in forming short nub-like processes. Because of these phenotypic changes, these activated microglia are known to be 'bushy' in appearance as described by Soltys et al. [27]. Large microglial cell bodies may account for the increase in proportional area seen within the intermediate lamina. However, using a phenotypic analysis, we show that microglial cell bodies within the intermediate lamina exhibit little change in cell body length between groups, suggesting that these cells are not being activated. This lack of microglial activation confirms that exercise alone does not exacerbate microglial activation within lamina VII and X. In mice after a SCI we would then expect the activation of microglia to relatively similar between animals who were given exercise and those who remained unexercised. The increase seen within the intermediate lamina must then be credited to either an increase in the number of microglia, or a change in the number/size of their processes. Numerical analysis of the microglia or their cell processes in these regions may help to distinguish the source of this proportional area increase.

Levels of EBD extravasation were found to be extremely high within lamina X of exercised animals. In lamina VII a smaller increase in BSCB permeability was seen. Surprisingly, these results suggest that exercise alone is able to increase the permeability of the BSCB in these regions. When the BSCB is weakened to this extent under pathological conditions, it may be dangerous for the CNS. Previously compartmentalized peripheral cells and molecules are now able pass through this barrier and into nervous tissue. The implications of these results may play an important role determining which exercise therapies would be most beneficial after a SCI. Because the BSCB is

already weakened after a SCI, certain task-specific exercises may further exacerbate this permeability. Perhaps the use of task-specific exercises with minimal impact on the strength of the BSCB should be of focus.

This increase in the permeability of the BSCB may be due to an increase in angiogenesis occurring in these regions. If neurons in lamina X and the intermediate lamina are becoming more activated in response to the exercise training, they will use up more oxygen, creating a potentially hypoxic environment. Low oxygen levels signal the need for new vasculature growth to supply the increased oxygen demand of the tissue [28]. This signaling is usually done through the expression VEGF. While VEGF is part of the angiogenesis signaling pathway, it is also a vascular permeability factor [29]. This VEGF induced angiogenesis results in a more permeable BSCB. It is very likely that this accounts for the increase in permeability that is seen in animals after exercise. Greater Ly6C labeling within lamina X supports this theory of angiogenesis with concomitant BSCB weakness. A slight increase in the proportional area of vasculature due to exercise alone was found in many exercised groups within the intermediate lamina, and all exercised groups in lamina X. This proportional area increase may be accounted for by the presence of additional blood vessels that grew into this region. Variation in the size of vasculature already present in the mice may also result in the increased proportional area seen, although it is unlikely. Larger vessels present prior to exercise training will result in a false positive, increasing the proportional area of vasculature attributed to angiogenesis. To compensate for this, measurements of the vessel's diameter may help to differentiate new from existing vasculature. Labeling of angiogenic markers such as VEGF or CD34 in future analysis may further support these findings.

The specificity of exercise delivered a variation in the type and dose of sensory cues to the spinal cord which had different effects on the microenvironment within the cord. Throughout analysis, the DHW group stood out, accentuated by its display of the greatest changes in microglia, vascular permeability, and amount of vasculature. The DHW group experienced a moderate delivery of eccentric cues to the CNS; a lower dose than in downhill running, however higher than the uphill exercise. The changes seen in the DHW group, suggest that this is the optimum dosage required to alter microglia, permeability, and vasculature in these regions. During walking exercise the duration of the eccentric contractions are longer, due to increased time in stance phase. While in running, the steps are quick, utilizing a short, intense signaling period. If this longer period of signaling led to increased activation in lamina X, it may explain the heightened demand for vasculature.

Unexpected findings from this study have sparked an interest in further investigation of its results. It remains unclear the reason for the increased level of microglia seen in the intermediate lamina of downhill walking animals. Measurement of the length of microglial processes or numerical evaluation of the microglia could shed some light on this question. The Ly6C labeling of the vasculature in lamina X strongly suggest angiogenesis as the cause for the BSCB permeability seen after exercise. However, this cannot be confirmed until further angiogenic markers are labeled and analyzed. Using VEGF or CD 34 to assess levels of angiogenesis may further strengthen this theory.

Conclusion

Delivery of eccentric signals in a dose dependent manner was found to differentially impact BSCB permeability as well as microglial reactivity. Downhill walking exercise significantly increased BSCB permeability as well as microglial reactivity when compared to sedentary animals. From this study we determined that downhill walking exercise was able to alter microglia within the intermediate lamina. The cause of these microglial alterations remains unclear. We also confirmed that microglia do not become phenotypically "activated" from task-specific

exercise as they are after SCI. An unexpected finding showed that exercise itself is able to increase the permeability of the BSCB within lamina X with a trend in the intermediate lamina. Further investigation suggests that for lamina X this is likely attributable to angiogenesis. These findings suggest that exercise plays a greater role in the microenvironment of the spinal cord than previously thought. Further investigation into these task-specific changes in microglia and BSCB permeability may reveal exercise conditions that area ideal for SCI recovery.

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